Regulation of miR-200c and miR-141 by Methylation in Prostate Cancer

Seodhna M. Lynch[1], Karla M. O'Neill[1,2], Michael M. McKenna[3], Colum P. Walsh[1], Declan J. McKenna[1]

[1]Biomedical Sciences Research Institute, Ulster University, Cromore Road, Coleraine, BT52 1SA

[2]School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, BT9 7BL

[3]Dept of Cellular Pathology, Western Health & Social Care Trust, Altnagelvin Area Hospital, Co. Derry, BT47 6SB

Introduction

In prostate cancer, many miRNAs are aberrantly expressed. This abnormal expression suggests that miRNAs are potentially promising in terms of diagnosis, prognosis and subsequent therapeutic intervention in this disease[1, 2].

miR-200c and miR-141 are co-expressed members of the miR-200 family, which play a key role in epithelial-to-mesenchymal transition (EMT) in cancer.

miR-200c and miR-141 are known to be abnormally expressed in several cancers, including prostate cancer [3, 4].

Epigenetic regulation of miR-200c via DNA methylation has also been reported in various cancers [5-7], but no studies to date have investigated this in prostate cancer.

We therefore proceeded to profile and correlate miR-200c/miR-141 expression and methylation status in prostate cell-lines and clinical prostate samples.

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Materials and Methods

Cell Lines: Non-malignant prostate epithelial cell-line RWPE1 and human prostate cancer cell-lines PC3, 22RV1 and LNCaP. Transfections performed at final concentration of 25nM.

Clinical Prostate Samples: Five 10μM sections were prepared from FFPE needle core biopsies (n=14) and FFPE prostatectomy biopsy samples (n=22) for RNA and DNA extraction using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Life Technologies). ORECNNI Ref: 10/NIR02/13.

PCR: miRCURY LNA™ microRNA PCR system (Exiqon, Denmark) used to measure miR-200c and miR-141 expression from 50ng (clinical samples) or 20ng (cell-line samples) template RNA. miRNA and gene expression performed on Roche LC480 Lightcycler.

Flow Cytometry: performed on Beckman-Coulter Gallios™ instrument using PI staining (cell cycle) or PI and Alexa® Flour 488-Annexin V Kit (apoptosis) (Life Technologies).

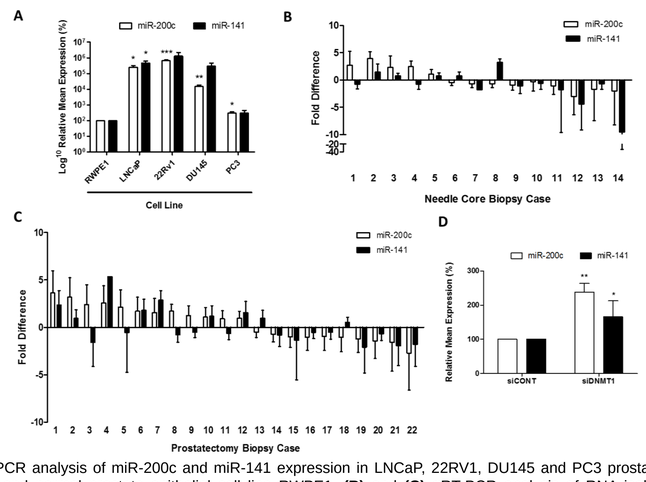
Aza and Genistein Treatment: PC3 cells were treated with decitabine (5-*aza*-2'-deoxycytidine) (1μM) for 72hours or genistein (40μM) for 120 hours. RNA and DNA extracted for analysis.

Pyrosequencing/COBRA: Bisulfite converted DNA was produced using the EpiTect Bisulfite Kit (Qiagen). The PyroMark™ Q24 pyrosequencer (Qiagen) was used to measure methylation levels within the miR-200c/miR-141 promoter region across CpG sites. Restriction enzymes BstU1 and Hinf1 was used in COBRA analysis.

Other Assays: XTT proliferation assay (Roche) and colony assay performed on transfected and control cells.

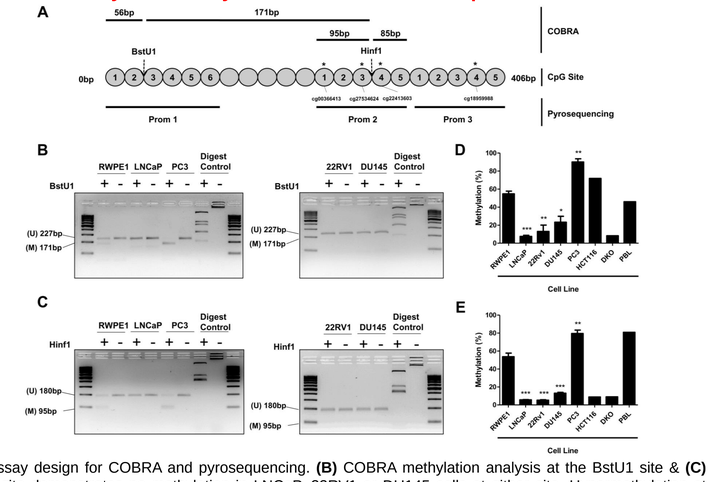
Results

FIGURE 1. Expression of miR-200c and miR-141 in PCa cells and tissues



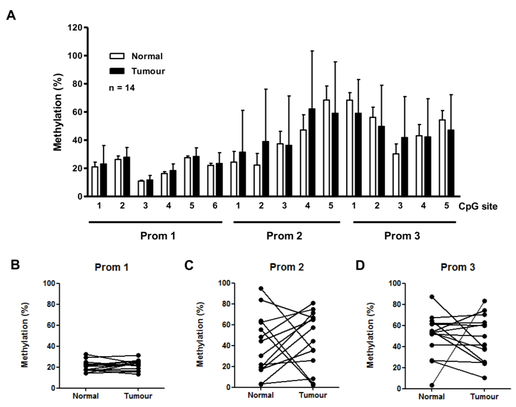
(A) qRT-PCR analysis of miR-200c and miR-141 expression in LNCaP, 22RV1, DU145 and PC3 prostate cancer cell lines and normal prostate epithelial cell line RWPE1. **(B)** and **(C)** qRT-PCR analysis of RNA isolated from needle core biopsy clinical specimens (n = 14) and prostatectomy biopsy clinical specimens (n = 22) showing fold change expression of miR-200c and miR-141 in individual tumour cases relative to matched normal tissue. **(D)** Demethylation treatment by knockdown of DNA Methyltransferase 1 (siDNMT1) in PC3 cells resulted in significantly increased expression of miR-200c and miR-141 (Student t-test p-values: *p<0.05, **p<0.01, ***p<0.001).

FIGURE 2. Methylation analysis of miR-200c/miR-141 promoter in PCa cell lines



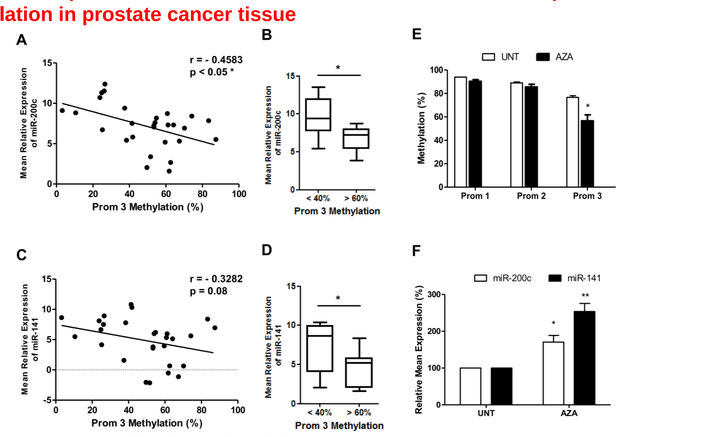
(A) Assay design for COBRA and pyrosequencing. **(B)** COBRA methylation analysis at the BstU1 site & **(C)** Hinf1 site demonstrates no methylation in LNCaP, 22RV1 or DU145 cells at either site. Hypermethylation at both sites in PC3 cells is indicated by digest products of 171bp and 95bp respectively. **(D)** Pyrosequencing of miR-200c/miR-141 promoter Region 1 (Prom 1) and **(E)** Region 2 & 3 combined (Prom 2 & 3) displays low methylation in LNCaP, 22RV1 cells and DU145, but significant hypermethylation in PC3 cells relative to normal RWPE1 cells. Control cells (HCT116; colon cancer cell line, DKO; fibroblasts with DNMT1 and DNMT3B knockout, PBL; peripheral blood lymphocytes). (Student t-test p-values: *p<0.05, **p<0.01, ***p<0.001).

FIGURE 3. Methylation analysis of miR-200c/miR-141 promoter in prostate cancer tissues



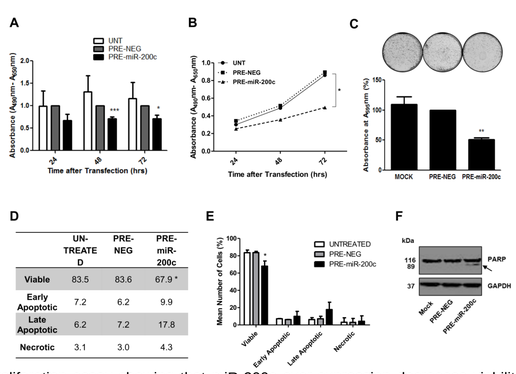
(A) Pyrosequencing analysis of DNA isolated from prostatectomy biopsy clinical specimens showing average methylation at 16 separate CpG sites within the miR-200c/miR-141 promoter in paired tumour samples relative to matched normal tissue (n = 14). Data represents mean ± SE of triplicate experiments. Below, the average methylation in all 14 paired tumour and normal samples is presented separately for **(B)** Prom 1, **(C)** Prom 2 and **(D)** Prom 3 regions of miR-200c/miR-141 promoter. Each dot represents the mean methylation percentage of all the pyrosequenced CpG sites in that region.

FIGURE 4. Expression of miR-200c and miR-141 correlates with promoter methylation in prostate cancer tissue



Mean expression of **(A)** miR-200c and **(B)** miR-141 shows a significant inverse correlation with the mean methylation percentage of the Prom 3 region of the miR-200c/miR-141 promoter. Cases with <40% methylation in the Prom 3 region showed significantly higher expression of **(C)** miR-200c and **(D)** miR-141 than those with methylation >60% in this region. **(E)** Treatment with decitabine (AZA) resulted in significant demethylation of CpG sites in the Prom 3 region and **(F)** up-regulation of miR-200c and miR-141. (Student t-test p-values: *p<0.05, **p<0.01, ***p<0.001).

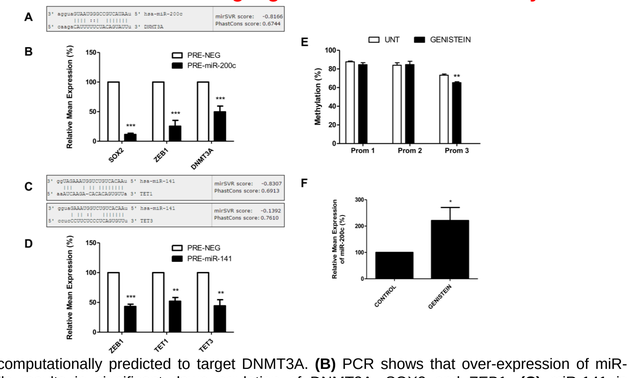
FIGURE 5. miR-200c over-expression inhibits cell growth and induces apoptosis



	UN-TREATED	PRE-NEG	PRE-miR-200c
Viable	83.5	83.6	67.9 *
Early Apoptotic	7.2	6.2	9.9
Late Apoptotic	6.2	7.2	17.8
Necrotic	3.1	3.0	4.3

(A) and **(B)** XTT Proliferation assay showing that miR-200c over-expression decreases viability of PC3 cells. **(C)** Representative images and quantification of crystal violet staining demonstrates that miR-200c over-expression significantly inhibits the colony formation ability of PC3 cells. **(D)** and **(E)** Quantification of Annexin V apoptosis assay showing that miR-200c over-expression induces apoptosis in PC3 cells. **(F)** Western blot demonstrating increase of PARP cleavage in miR-200c transfectants compared to control cells. (Student t-test p-values: *p<0.05, **p<0.01, ***p<0.001).

FIGURE 6. miR-200c and miR-141 target genes involved in cell methylation



(A) miR-200c is computationally predicted to target DNMT3A. **(B)** PCR shows that over-expression of miR-200c in PC3 cells results in significant down-regulation of DNMT3A, SOX2 and ZEB1. **(C)** miR-141 is computationally predicted to target TET1 and TET3. **(D)** PCR shows that over-expression of miR-141 in PC3 cells results in significant down-regulation of TET1, TET3 and ZEB1. **(D)** Pyrosequencing analysis shows treatment of PC3 cells with genistein (40μM daily for 7 days) results in significant demethylation of miR-200c/miR-141 promoter region 3 (Prom 3). **(F)** In the same genistein-treated PC3 cells, miR-200c expression is significantly increased as measured by PCR. (Student t-test p-values: *p<0.05, **p<0.01, ***p<0.001).

Summary and Conclusion

- ❖ This is the first study to show a correlation between DNA methylation and expression of miR-200c and miR-141 in clinical prostate specimens.
- ❖ Our findings provide evidence that expression of miR-200c and miR-141 is regulated by methylation of CpG sites in their promoter in PCa cells.
- ❖ Profiling their expression and methylation status may therefore have potential as a novel biomarker in the diagnosis and prognosis of PCa.
- ❖ Our data also suggests that aberrant miR-200c/miR-141 expression will affect DNMT3A and TET genes, key components of the methylation machinery which will influence global cellular methylation levels.
- ❖ Furthermore, we propose that manipulation of miR-200c and miR-141 expression by epigenetic alterations, either through chemical or dietary means, may be the basis for a possible therapeutic intervention for this disease.

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ADDRESS FOR CORRESPONDENCE

Dr Declan McKenna, Ulster University, Coleraine, N. Ireland, UK, BT52 1SA

E.-mail dj.mckenna@ulster.ac.uk Tel. +4428 7012 4356

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